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Epidemiology and Identification of Actin Gene of *Trichomonas vaginalis* Genotypes in Women of Southeast of Iran Using PCR-RFLP

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Abstract

Objectives: C Trichomoniasis is one of the most common sexually transmitted diseases in the world, which is caused by *Trichomonas vaginalis*. It is also the most commonly reported sexually transmitted disease after the viral infections, which affects around 180 million people around the world each year. The people infected with this parasite exhibit a wide range of symptoms. To the best of our knowledge the genetic variation, prevalence and related factors affecting the disease have not been well studied. Therefore, this study aimed to determine the prevalence of *T. vaginalis* in women of southeast of Iran.

Materials and Methods: Out of 500 patient women referred to the hospitals of Imam Khomeini in Zabol and Ali Ibn Abi Talib (AS) in Zahedan, 25 positive clinical samples were isolated from vaginal discharge and urine by culture method during June 2015 and May 2016. First, DNA was extracted and then all samples were subjected to nested PCR. Six different genotypes of actin gene were identified by PCR-RFLP in *Trichomonas vaginalis* in Zahedan and Zabol. All PCR products were digested with *HindII, RsaI,* and *MesI* restriction enzymes. All participants completed a questionnaire recommended by gynecologists and midwifery experts.

Results: As a result, the genotypes of H, G, E, I, and N were identified in this study, from which the genotype E was the dominant genotype of *T. vaginalis* in Zahedan and Zabol. There was also a significant association between the type of clinical symptoms and the level of infection (P=0.0001).

Conclusions: To sum up, disease as a health problem must be controlled through epidemiologic and genetic methods. Moreover, controlling the disease is closely associated with education and drug resistance or sensitivity related to genetic variation and epidemiologic factors.

Keywords: *Trichomonas vaginalis*, Actin gene, Zahedan, Zabol, Epidemiology

Introduction

Trichomoniasis is one of the most common sexually transmitted diseases in the world, which is caused by *Trichomonas vaginalis*. It is also the most commonly transmitted infectious disease after viral infections (1). The parasite lives in a woman's vagina, especially the cervix, and in the urethra, and in men's prostate gland (2). In women, infection with *T. vaginalis* can cause vaginal, cervical, and urethral inflammations (3,4). Clinically, not only the establishment of the parasite in the vaginal wall does not cause clinical symptoms, but also many physiological and pathological factors such as estrogen, glycogen, and pH of the vagina are effective in the growth and pathogenicity of trichomoniasis (5,6). The parasite grows better in a humid environment, in a pH range of 4.9 to 7.5 and temperature of 35°C to 37°C, and if these conditions are less or greater than the desired level, the organism is destroyed (7). *T. vaginalis* can be transmitted to the infant during the labor and passing the baby through the birth canal. The infection can be observed as pneumonia or conjunctivitis in infants who are infected during birth (8). It is assumed that male genital oxidative properties can inhibit certain pathogens from the parasite. For example, the Zinc element in the prostate fluid has a cellular poisoning effect (cytotoxicity) on the parasite (9).

The World Health Organization (WHO) estimates the presence of trichomoniasis in 180 million people, which is the cause of 10% to 25% of vaginal infections (10, 11). In Iran, its prevalence has been reported in various groups between 0.5% and 30% (12). It is estimated that around 10% to 50% of *T. vaginal* infections in women are asymptomatic, and this percentage can be higher in men (4). The prevalence of asymptotic infection with the parasite in sexually active women has been reported in the United States as 2-3 million per year (13).

Relatively extensive researches have been conducted on various aspects of trichomoniasis, including epidemiology, diagnosis, and clinical manifestations. However, little information is available on genetic traits and its relevance to the phenotypic characteristics of this parasite (14).

Several methods have been used to identify the gene or a fragment of gene in the genetic studies of *T. vaginalis*

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including characterization of antigenic properties, isozyme studies, proliferation of random polymorphic DNA fragments (RAPD = Random Amplified Polymorphic DNA), genomic DNA hybridization, sequence analysis of parts of ribosomal genes (sequencing), and restriction fragment length polymorphism (RFLP). Actin gene, ITS, Beta-tubulin, 18s ribosomal gene fragment, and the protein kinase are the most widely used parts of the *T. vaginalis* genome in the diagnosis (15,16).

Actin is one of the most important and abundant molecules in eukaryotic cells. This protein plays an important role in the movement, phagocytosis, cellular adherence, transaction signal, and a large number of vital activities in microorganisms, as one of the main components of the cytoskeleton filaments (17). The actin protein in *T. vaginalis* is coded by a 9-membered family, with nucleotide similarities in 5 genes similar to the actin gene in the mammals. Transformation of *T. vaginalis* from a tetanus form to the amebic form, and formation of an appendage such as pseudopodia occurs when the parasite is attached to the host cell surface. The ability of this deformation and attachment to the host cell seems to be related to the pathogenicity of the parasite. Therefore, actin gene can play an important role in the pathogenicity of the parasite (18,19), due to the role of actin in changes in the morphology of the parasite, as a target for the PCR-RFLP method.

Materials and Methods

Isolation of *Trichomonas vaginalis* and Locale of the Study

Sistan and Baluchistan is located in the Southeast of Iran, and Zabol in the North of this province has a hot and dry desert climate. Zabol lies on the border with Afghanistan (20). Zahedan, located near Pakistan and Afghanistan, has an estimated population of 567 449 (21).

A total of 500 women referred to Ali Ibn Abi Talib Hospital in Zahedan and Imam Khomeini hospital in Zabol during June 2015-May 2016 were investigated. First, 25 specimens were detected by a positive culture. The specimens cultured in Dorset culture medium at 37°C for 72 hours were tested for positive samples of the parasite and stored at -20°C for molecular investigations.

This study was performed on 500 women in 2 groups including 150 women referred to Imam Khomeini hospital in Zabol city and 350 women referred to Ali Ibn Abi Taleb Hospital in Zahedan, during June 2015 to May 2016. Participants were from a wide range of ages from 18 to 70 years old and their education level varied from highly educated to illiterate. The participants were also investigated in terms of occupation, age, place of residence, education level, and residence status. The results of this study are presented in two sections based on the objectives of the study.

First, having made the necessary adjustments with the clinic of the Zabol Imam Khomeini hospital and Zahedan Ali Ibn Abi Talib hospital, as well as obtaing consent from the patients, vaginal specimens of the women were examined by a physician or midwifery expert using two sterile swabs under the supervision of the Zabol and Zahedan Universities of Medical Sciences. One of the swabs was quickly transferred to the culture medium under sterile conditions, and the other swab containing the specimen was placed in a transport medium, including sterile physiological serum or sterile ringer serum in twisted tubes of 100 × 16 mm, sterile. However the urine specimen, after diagnosis through the Urinalysis laboratory procedure, was immediately transported to the laboratory in order to do the microscopic examination.

Samples were immediately placed in an incubator at 37°C upon arrival, and in the case of urine specimens, the sediment was examined under a microscope after centrifugation. Proliferous flagellated specimens were considered as positive; however, all suspicious specimens were cultured in Dorset medium and kept up to 72 hours or up to 96 hours in an incubator and discarded if the parasite was not observed.

After culture in Dorset medium (to maintain the parasite), they were kept in a freezer for the molecular testing. In addition, each patient had a questionnaire and one related number.

DNA Extraction

Frozen samples were removed from the freezer for PCR, and after melting were first centrifuged for 5 minutes at 5000 rpm. Then, DNA was extracted using the extraction kit (Yekta Company, Iran). Afterward, the entire PCR product was digested by *HindII, RsaI,* and *MesI* restriction enzymes.

PCR-RFLP

In this method, the effects of endonucleases (restriction enzymes) are used to differentiate Trichomonas strains. The actin gene was amplified by nested PCR, using two pairs of specific primers (16). External primer pair included:

Tv8s (5'-TCT GGA ATG GCT GAA GAA GAC G -3 ') and Tv9R (5'- CAG GGT ACA TCG TAT TGG TC-3) and a pair of internal specific primers: Tv10s (5' -CAG ACA CTC GTT ATC G-3') and Tv11R (5'- CGG TGA ACG ATG GAT G-3').

The target gene had a length of 1100 bp. Since nested PCR was used, the first stage product was a template for the second PCR. The second stage was similar to the one conducted and only primers of F2 and R2 should have been used instead of primers of F1 and R1, and also instead of the original DNA, the product of the first step with a volume of 1 µL should have been added.

The First Stage of PCR

The PCR components present in each microtube with a volume of 0.2 mL for the reaction volume of 25µL were as follows: master mix 12.5 µL, forward primer (F_1) 1 µL,

reverse primer (R_1) 1 µL, ddw 9.5 µL, and DNA 1 µL. PCR was performed as follows: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 3 minutes; and final extension at 72°C for 7 minutes.

Second Stage of PCR

The PCR components present in each microtube with a volume of 0.2 mL and a reaction volume of 25 µL were: master mix 12.5 µL, forward primer (F_2) 1 µL, reverse primer (R_2) 1 µL, and DNA (diluted) 1 µL.

In the second stage of nested PCR, after Master Mix preparation, the samples were placed in the thermal cycler. This stage was different from the first stage only in that annealing temperature was set at 55°C instead of 61°C. After completing the second stage, the PCR product was electrophoresed on 1% agarose gel and the desired band was compared to a 100 bp DNA ladder (the target band was about 1100 bp in size).

In the second stage, PCR protocol was followed as: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 61.3°C for 30 seconds, extension at 72°C for 3 minutes; and final extension at 72°C for 7 minutes.

Results

Frequency of Infection With *Trichomonas vaginalis*

Results of a Direct Test of Samples

In the study of wet cultures from 500 samples of urine and 500 vaginal samples tested using the direct method, 20 samples were diagnosed as positive in terms of the presence of *T. vaginalis* via observing the alive moving parasites in the specimen figure (Figure 1). Using this method, the prevalence of *T. vaginalis* in the samples from Ali Ibn Abi Talib hospital in Zahedan was 70% (14 positive samples), and in the samples from Zabol Imam Khomeini hospital was 30% (6 positive samples).

Results of Experiments on Dorset Medium

Samples cultured in the Dorset medium were tested on a daily basis for 72 hours and positive specimens were confirmed with alive moving parasites. Moreover, 25 samples from all 500 vaginal and urine swab specimens were diagnosed as positive in terms of the presence of *T. vaginalis*. The results of the distribution of the parasite related to direct smear and culture media are shown in Table 1.

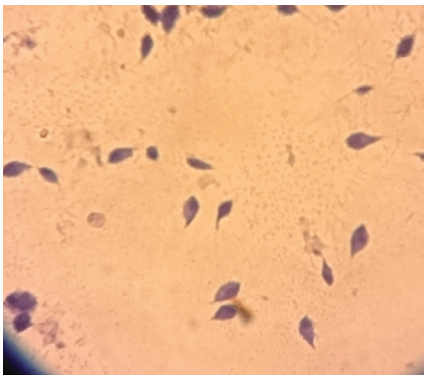

Figure 1. Trophozoite of *Trichomonas Vaginitis* in Dorset Culture Medium Dyed With Giemsa.

Age-Related Results of the Participants

The results of the present study showed that the youngest and the oldest ages of infection were 25 and 50 years, respectively. The oldest age of infection was between the ages of 33 and 40 (in Zabol). While in Zahedan, the youngest infection age and the oldest age of infection were 25 and 53 years, respectively.

Results Related to the Education Level of the Participants

Most of the women surveyed in this study (334 women) had a diploma or less than a high school diploma. However, there was no significant association between the education level and infection rate (*P* value = 0.329, Figure 2).

Results Based on the Occupation of the Participants

Many of the surveyed women in this study were housewives (431 cases), and 92% of the women infected with *T. vaginalis* (23) were housewives. No significant association, however, was seen between the rate of housewives and infection (*P* value = 0.388, Figure 3).

Results Based on the Place of Residence of the participants

Most of the women surveyed in this study lived in urban areas with a population of 416 (83.2%). There was no statistically significant relationship between the distribution of the population in urban and rural areas and infection rate (*P* value =0.323; Figure 4).

Age-Related Results

In terms of infection, the highest rate of infection was related to the age group of 30-40 years old and then the age group of 40-50 years old, and the severity of infection was lower in the age group of 25-30 years old. Moreover, infection was not observed among the participants under

Table 1. Prevalence of Trichomoniasis Infection in the Participants of the Study Based on the Diagnostic Method

Diagnostic Methods	Study Location	No. of Positive Samples	No. of Negative Samples
Direct smear	Ali Ibn Abi Talib hospital of Zahedan	14	336
	Imam Khomeini hospital of Zabol	6	144
Dorset culture medium	Ali Ibn Abi Talib hospital of Zahedan	18	332
	Imam Khomeini hospital of Zabol	7	143

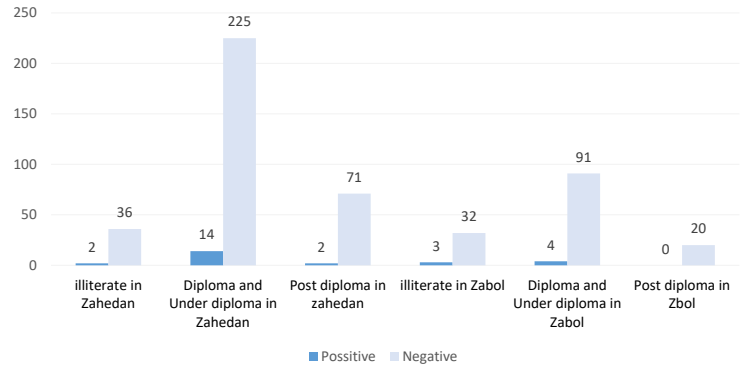

Figure 2. Prevalence of Trichomoniasis Infection Among the Participants in Terms of Education Level.

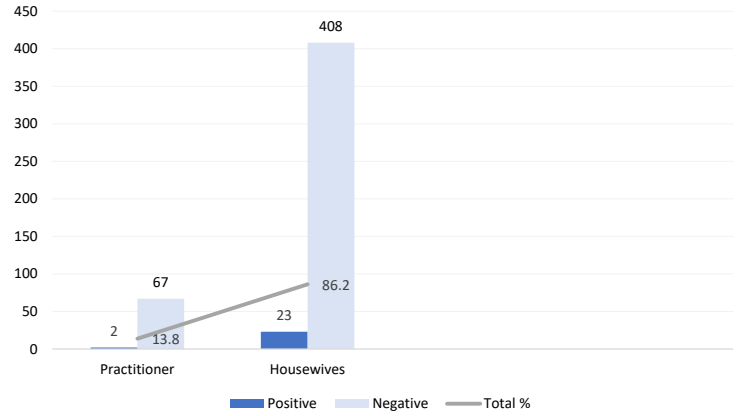

Figure 3. Prevalence of Trichomoniasis Infection Among the Participants in Terms of Occupation.

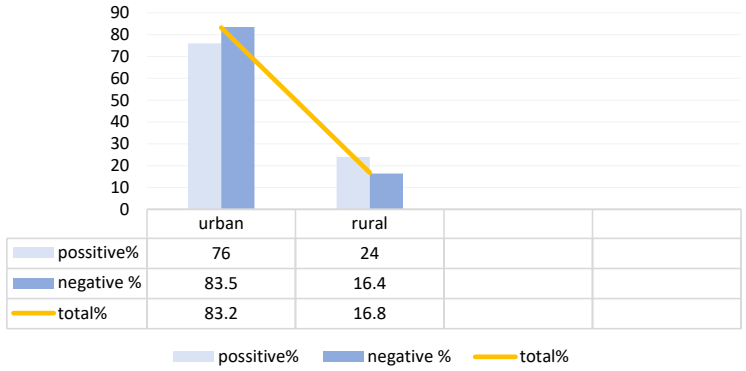

Figure 4. Prevalence of Trichomoniasis Infection Among participants Based on Their Place of Residence.

25 years and over 54 years. In Zahedan and Zabol, the mean ages were 37.37 and 39.35 years, respectively.

Contraceptive-Related Results

A total of 192 participating women in this study did not use any contraceptive method. Out of 25 patients infected with *T. vaginalis*, 20 (80%) were females (Figure 5).

Results Based on the Number of Deliveries

A total of 179 women surveyed in this study had two or fewer deliveries, with a positive rate of 7.8%. Prevalence of trichomoniasis was statistically different among participants in terms of the number of deliveries (*P* value=0.03; Figure 6).

Results of the Type of Symptoms and Infection With *Trichomonas vaginalis* in the Subjects Participating in the Study

Yellow discharge as a sign of trichomoniasis was seen in 13 patients (52%) infected with *Trichomonas vaginalis,* from a

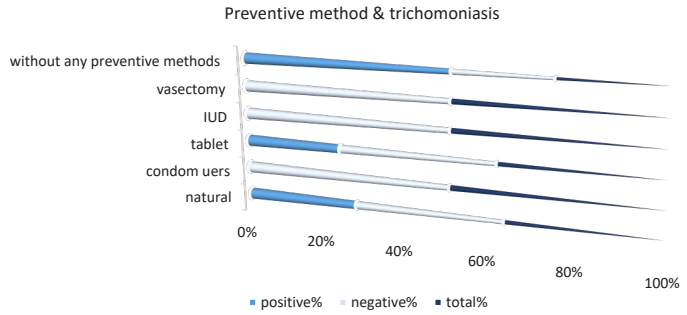

Figure 5. Prevalence of Trichomoniasis Infection Among Participants in Terms of Contraceptive Methods.

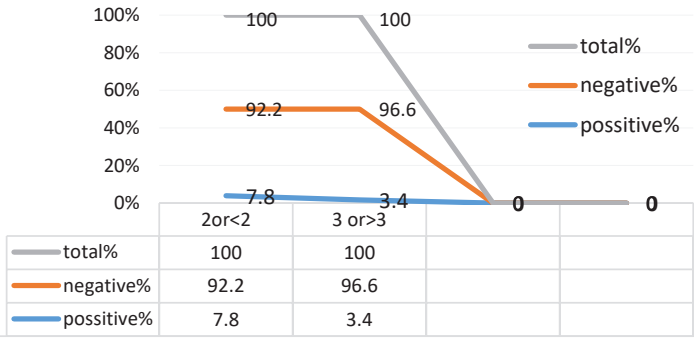

Figure 6. Prevalence of Trichomoniasis Infection Among Participants in Terms of the Number of Deliveries.

total number of positive cases (25). There was a significant association between the type of clinical symptoms and the level of infection (P=0.0001; Figure 7).

The nested PCR test resulted in the amplification of fragments of 1100 bp from the actin gene nucleotide sequence (Figure 8a and 8B). The Nested PCR assay could reproduce the desired nucleotide fragments in all 25 extracted DNA sequences in a single-banded and identical length. Table 2 shows the different patterns and sizes of the enzyme cleavages (16).

HindII restriction enzyme (Hindi): two electrophoretic patterns are obtained by digestion with this enzyme. In a model, there are three bands including 827 pb, 213 pb, and 60 pb. And in another model, there are 426 pb, 401 pb, 213 pb, and 60 pb bands, while 213 pb and 60 pb bands are characterized in all patterns. At least four electrophoretic patterns are obtained using the *RsaI* enzyme digestion. Bands of 236 pb and 106 pb are found in all patterns. The difference between the other patterns is in the presence or absence of bands of 568 pb, 452 pb, 190 pb, 116 pb, 103 pb, and 87 pb. Three types of electrophoretic bands are obtained by digestion with the *MesI* enzyme. The band of 581 pb is found in all patterns; 581 pb and 519 Pb bands are observed in one pattern, and in another pattern, 581

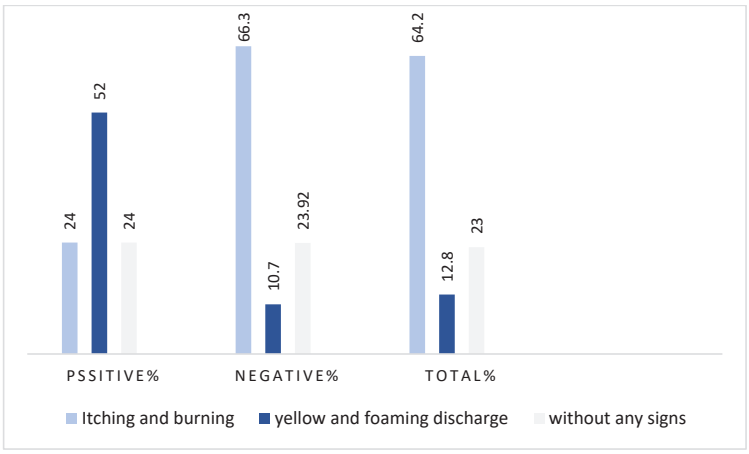

Figure 7. Prevalence of *Trichomonas vaginalis* (Positive Cases) According to Clinical Symptoms.

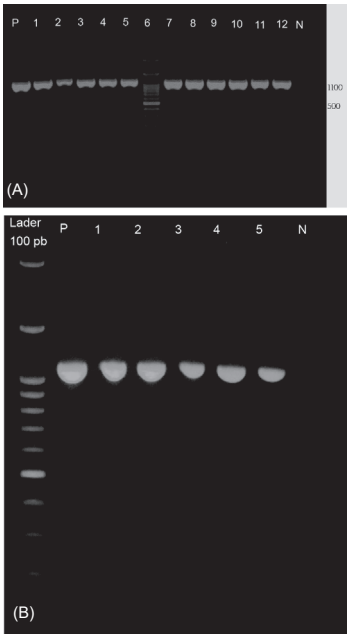

Figure 8. (A) PCR Product of the Actin Gene that Produces the Resulting Band with an Equivalent Weight of 1100 pb. ***Note***. P: positive control, Lanes 1-5: the number of positive samples of *Trichomonas vaginalis*, Lane 6: 100 bp DNA ladder, Lanes 7-12: patient samples, N: negative control.
(B) ***Note***. Lane 1: 100 bp DNA ladder, Lane 2: positive control (P), Lanes 1-5: the number of positive samples of *Trichomonas vaginalis* in the patient samples, N: negative control.

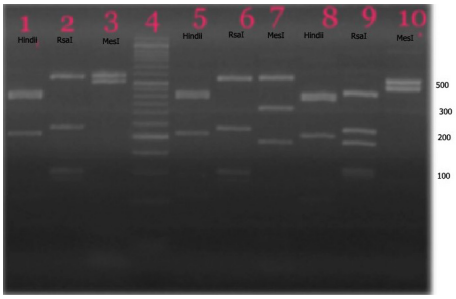

Figure 9. RFLP Result of Actin Gene (Genotypes H, N, and I) Using the Restriction Enzymes of *HindII* (HincII), *MesI*, and *RsaI*. ***Note***. Lanes 1, 5, and 8 are the products of the HincII enzyme, lanes 2, 6, and 9 are produced by *RsaI* enzyme, and lanes 3, 7, and 10 are the products of the *MesI* enzyme. Lane 4 is DNA ladder with the length of 50 pb, lanes 1, 2, and 3 belong to genotype H, lanes 6, 5, and 7 belong to genotype N, and lanes 9, 8, and 10 belong to genotype I.

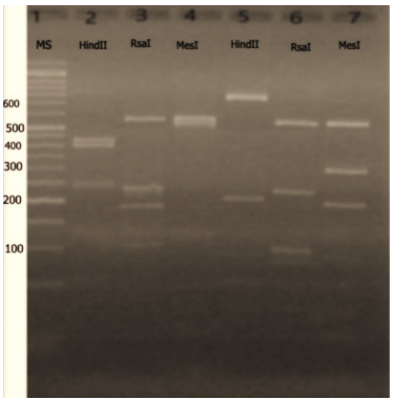

Figure 10. RFLP Result of Actin Gene (Genotypes G and E) Using Restriction Enzymes of *HindII* (HincII), *MesI*, and *RsaI*
Note. Lane 1: DNA ladder (50pb), lane 2: product of the *HindII* (HincII) enzyme, lane 3: product of the *RsaI* enzyme, and lane 4: product of *MesI* enzyme. Lane 5, product of the HincII enzyme () belonging to genotype G; lane 6, product of the *RsaI* enzyme and lane 7, product of the *MesI* enzyme belonging to genotype E.

pb, 315pb, and 204 pb are observed; and the next pattern includes 581 pb, 333 pb, and 186 pb (Figures 9 and 10). Table 3 shows the frequency of trichomoniasis infection among the participants of this study in terms of genotype.

Results of Dominant Genotype With Clinical Signs of Trichomoniasis Infection in the Subjects

Most women infected with *T. vaginalis* had genotype E. Nearly all of them had yellow and frothy discharge. Genotype E had a more prevalence (50%) than other genotypes including N, H, G, and L. There was a significant association between the dominant genotype and the incidence of symptoms (*P* value =0.0001, Figure 11).

Discussion

In this study, a total of 500 women were screened, from which 150 lived in Zabol city. Using wet smear method and culture method, 6% and 7% were diagnosed as positive in terms of vaginal trichomoniasis, respectively. In Zahedan, among 350 patients, 14 cases (4.9%) using the direct method and 18 cases (5.1%) using the culture method were diagnosed with the infection, while the prevalence of trichomoniasis in Zabol and Zahedan was determined as 4.2% and 1.5%, respectively, using PCR-RFLP method.

Table 2. Different Patterns and Sizes of the Enzyme Cleavages by *HindII*, *RsaI*, and *MesI* Restriction Enzymes, and Genotypes of the Actin Gene of *Trichomonas vaginalis*

Genotype	Restriction With *HindII* (Pb)	Restriction With *RsaI* (Pb)	Restriction With *MseI* (Pb)
A	827-213-60	568-236-190-106	581-519
E	827-213-60	568-236-106-103-87	581-315-204
G	426-401-213-60	568-236-190-106	581-519
H	426-401-213-60	568-236-106-103-87	581-519
I	426-401-213-60	452-236-190-116--106	581-519
M	426-401-213-60	568-236-190-106	581-333-186
N	426-401-213-60	568-236-106-103-87	581-333-186
P	426-401-213-60	452-236-116--106-103-87	581-333-186

Table 3. Prevalence of Trichomoniasis Infection among the Participants Based on the Genotype

Genotype	Place Study			
	Zahedan		Zabol	
	No.	(%)	No.	(%)
I	2	11.1	0	0
E	7	38.9	5	71.4
G	5	27.7	2	28.6
H	1	5.6	0	0
N	1	5.6	0	0
Mix (G/I)	2	11.1	0	0

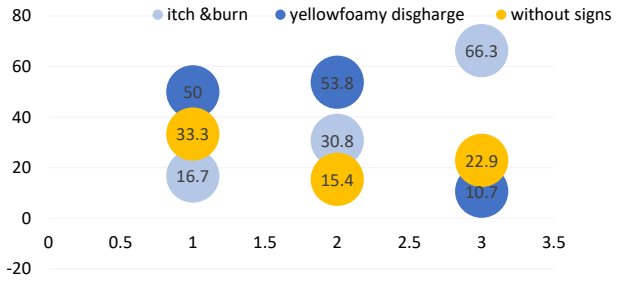

Figure 11. Results of Dominant Genotype With Clinical Signs of Trichomoniasis Infection in the Participants.

Trichomoniasis infection is increasing in developing countries and in high-risk groups (22). In line with the present study, in a study conducted in 2010 on women referred to the women's clinic in a gynecological hospital in Greece, the prevalence of *T. vaginalis* was reported as 4.6%, using wet mount, and culture method, and PCR from which culture method was more sensitive (23).

According to various studies carried out in Iran, the incidence of infection in Iran is between 0.5% and 30%. The highest prevalence was among the groups referred to the sexually transmitted diseases clinics, as well as the higher rates of infection in women prisoners, prostitutes, and those with a lower level of health (23).

Recently, along with the development of DNA-based laboratory methods, molecular methods have also been developed to detect organisms, including *T. vaginalis*. These methods are privileged to other diagnostic methods due to their high sensitivity and specificity. Various molecular methods have been used to identify and detect *T. vaginalis* parasites, from which molecular methods of PCR, nucleic acid hybridization, and real-time PCR have been reported more sensitive compared to direct and culture methods. Using the PCR method is more common than other methods in diagnosis, and this is true about the diagnosis of vaginal trichomoniasis.

In this study, the Nested PCR assay was performed for actin gene and then PCR specimens were digested with restriction enzymes including *HincII*, *RsaI*, and *MseI* (Fermentas). As a result, genotypes of H, G, E, I, and N were obtained in this study. According to the results of digestion with the enzymes and different alleles, and also according to the identification protocol of Table 1, 25 cases infected with *T. vaginalis* were identified in Zabol. There were 5 samples with genotype E and 2 samples with genotype G from 7 positive samples. In Zahedan, from 18 positive samples, 2 samples with genotype I, 7 samples with genotype E, 5 samples with genotype G, 1 sample with genotype H, 1 sample with genotype N and 2 samples with the mixing genotype (I and G) were determined. However, the dominant genotype in Zahedan and Zabol was determined as genotype E. At last, three samples were sent for the sequencing.

In 2008, Crucitti et al (24) conducted a survey on 151 positive samples referred to health centers in the Democratic Republic of Congo and Zambia. The three isolates of reference number 30001, 3024, and 50141 for the isolation of *T. vaginalis* and determination of parasite genotypes were assessed by PCR-RFLP using the actin gene. First, nested PCR was used with two external primers: TV8s (ops), TV2r, and internal TV10S (IPS) and TV11R PCR primers. Then, 3 *HindII, RsaI,* and *MesI* restriction enzymes were used for RFLP. Results showed that at least 8 genotypes were found in the actin gene. Three reference isolates (30001, 3024, and 50141) had also different genotypes of H, G, and E, respectively. Sixty-one samples were from Congo and 90 samples were from Zambia. The dominant genotypes in Kongo and Zambia were E (57%) and G (47%), respectively (18). In this study, genotype E was the most common genotype. Moreover, 5 genotypes were found in the actin gene, described by Crucitti, et al (24).

In a study by Tavakoli Oliaee et al (25) during 2012-2013, 85 samples of suspected cases referred to health centers in Kerman and 70 samples from Shiraz were examined in order to isolate *Trichomonas vaginalis* and determine its genotypes in patients with trichomoniasis using PCR-RFLP. After examining the pattern of fragments obtained from RFLP, 6 different genotypes including H, G, E, I, M, and N were identified. The dominant genotypes in Kerman and Shiraz were H (50%) and I (37.5%), respectively. The results of our study corroborated the aforementioned results.

In a study by Ziaei Hezarjaribi et al (26) during 2012-2013, PCR-RFLP method was used in order to identify and determine the genotype of *T. vaginalis* isolates in women referred to health centers of Sari. After examining the pattern of fragments obtained from RFLP, 3 different genotypes including E, G, and I were identified, from which genotype E was the dominant Genotype. Based on the protocol, 8 isolates of *T. vaginalis* were identified through screening activities and 4 isolates were disabled by smear and Pap smear; 6 samples were identified with genotype E and 4 samples with genotype G and 2 samples with genotypes I. Moreover, the prevalence of respiratory

infections was assessed in 500 patients, from which 7 cases (1.4%) were identified using a wet spreading method and 8 cases (1.6%) were identified using a positive culture. The results of Ziaei Hezarjaribi et al was not in agreement with the results of the present study.

Relatively extensive research has been done on various aspects of trichomoniasis, including epidemiology, diagnosis, and clinical manifestations. However, little information is available about genetic traits and its relevance to the phenotypic characteristics of this parasite. Among the methods used for genetic studies of *T. vaginalis,* characterization of antigenic properties, isozyme studies, proliferation of random polymorphic DNA fragments (RAPD), genomic DNA hybridization, sequence analysis of parts of ribosomal genes (sequencing), and RFLP can be referred to. These methods are used for identifying the gene or a gene fragment. ITS, actin gene, beta-tubulin, 18s ribosomal gene fragment, and the protein kinase are the most widely used parts of the *T. vaginalis* genome in the diagnosis (27,28).

Conclusions

Trichomoniasis is a common sexually transmitted disease in the southeast of Iran. The prevalence rate was estimated 5%. In diagnosing the disease, we found culture method more sensitive than the direct method. Further, our results demonstrated a wide genetic variation of the parasite in this region, however the most predominant genotype was E.

Limitations of the Study

Due to the emigration of some participants or changed addresses, and not referring to the hospital or deaths, some patients contributing to the study were excluded.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The research followed the tenets of the Declaration of Helsinki. Consent was also obtained from all participants. All patients took part in this study voluntarily. Further, this study was approved by the Ethics Committee of Zabol University of Medical Sciences (ethical code: zbmu.1.Rec.1395.44).

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